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Studies on the Prodigiosin Production from *Streptomyces coelicolor* in Liquid Media by Using Heated *Lactobacillus rhamnosus*

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ABSTRACT

Prodigiosin (PG) is not only an antibiotic but it has also been characterized immunosuppressive and anticancer activities. Prodigiosin is produced by *Streptomyces coelicolor* and *Seratia marcescens*. In this study, prodigiosin (PG) production of *S. coelicolor* was optimized using 50µL, 100µL of heated cells at 70°C and 100°C of *Lactobacillus rhamnosus*. With 50µL heat-killed cells at 70°C *L. rhamnosus*, prodigiosin (PG) production of *S. coelicolor* enhanced the maximum prodigiosin concentration (9.79 ± 1.68 mg/L) by six-fold at the early stage in the second day incubation in the comparison with prodigiosin (PG) production of *S. coelicolor* without heated *L. rhamnosus* (1.43 ± 0.22 mg/L). There were the affects of the components of *L. rhamnosus* on PG production. This is the first report that *L. rhamnosus* affecting on PG production in *S. coelicolor*.

INTRODUCTION

Prodigiosins (PGs) are the secondary metabolites produced by *Serratia* and *Streptomyces* genera (William 1973). For several decades, PGs show numerous biological activities, including antibacterial, antifungal, antiprotozoal (Mekhael *et al.*, 2009), antimalarial (Isaka M *et al.*, 2002), immunosuppressive (Han *et al.*, 2001), anticancer (Soto *et al.*, 2004; Monato and Pelrez, 2001), and dyeing of silks and wools (Alihosseini *et al.*, 2008) properties. PGs belong to a family of naturally occurring tripyrrole bright red pigments. *Streptomyces coelicolor* was known as safe microorganism which produces at least four kinds of antibiotic; namely actinorhodin, methylenomycin (Wright and Hopwood, 1976), the calcium-dependent lipopeptide antibiotic (CDA) (Ruth an Howood, 1980; Kempter *et al.*, 1997), and an analogue of prodigiosin called undecylprodigiosin (Luti and Mavitna, 2011).

In the industrial point of view, the optimal conditions in order to increase the production of PG are necessary due to its potential application in future. Starting from the actual situation, a new strategy was applied to increase the production of PG on *Streptomyces coelicolor* recently; it is the use of dead cells bacteria as factors that increase the production of PG on *S. coelicolor* (Luti and Mavituna, 2011; Rigali *et al.*, 2008). Some reports shown that N-acetyl glucosamine, one of main components of peptidoglycan, may trigger antibiotic production in *Streptomyces* (Tsao *et al.*, 1985). For this reason, Lactic acid bacteria (LAB) - a group of gram-positive bacteria and considered as commercial microorganisms with no pathogenic potential which has a thick peptidoglycan layer containing the activity on N-acetyl glucosamine and membrane products may play a new role as an elicitor in case of PG production in *S. coelicolor*. In those regard, the aim of this study was therefore to develop the production of PG on *S. coelicolor* using the heated *Lactobacillus rhamnosus* LGG.

MATERIALS AND METHODS

Microorganisms and growth cultures

Streptomyces coelicolor was obtained from John Innes Centre as a gift. *Lactobacillus rhamnosus* LGG was isolated and identified in yogurt inside Vietnam. For *Streptomyces coelicolor*, YEME media were used. For *Streptomyces coelicolor* inoculation, a stock of 10^8 spores was used.

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A loopful of *Lactobacillus rhamnosus* was inoculated in a test tube containing 5ml MRS broth medium. It was incubated at room temperature for 2 days. Then the bacterial cell suspensions were treated at 50°C, 70°C, and 100°C in 1 h before adding in the *Streptomyces coelicolor* culture. The morphology of these strains was tested, using gram stain.

Prodigiosin production tests

For study on prodigiosin production in medium, 20 µL of *Streptomyces coelicolor* prepared stocked were cultured in a shaken flask at 28°C with shaking at 200rpm in 48 hours. The experiments were carried out in triplicate and the results represented in the arithmetic average.

After 48 hours, 0.2 ml of inoculums was added into new flasks containing 20 mL medium. At this step, the different amounts of heated *L. rhamnosus* suspension were added, using a volume of 50 µl or 100 µl of *L. rhamnosus* heated at 70°C and 100°C. The flasks were incubated at 28°C with a shaking at 200 rpm. Every day, one ml of each culture was taken for the analyses.

Isolation of prodigiosin

All the cultures were harvested by centrifuging at 10,000 rpm for 10 minutes. The supernatant (20 mL) and pellet (2 g) were harvested. The prodiogiosin extraction was done with a modification (Helvia *et al.*, 2010). For the supernatant, the pigment was extracted by using chloroform with an equal amount to supernatant. The chloroform fraction was extracted by using methanol with an optimal ratio (1:1).

Further methanol extraction was done for a complete extraction. All methanol layers were collected to evaporate until dryness and the amount pigment obtained on a dry weight basis was calculated. For the pellet, the methanol (10 mL) was used for extraction. Further methanol extraction was done for a complete extraction. All methanol layers were collected to evaporate until dryness and the amount pigment obtained on a dry weight basis was calculated. The calculated amount of pigment obtained in the pellet and supernatant of culture gave the total amount of prodigiosin produced in *Streptomyces* in different condition. The analyses of results were performed by one-way ANOVA on SPSS 16.0 software.

RESULTS AND DISCUSSION

Temperature effects on *Lactobacillus*

The survival of *Lactobacillus* was checked after heated at 50°C, 70°C and 100°C. The heated *Lactobacillus* samples were spread on MRS agar which were incubated at room temperature from 48 h to 120 h. There were the colonies appearing on the MRS agar in case the *Lactobacillus* was heated at 50°C. In order to confirm the *Lactobacillus* colonies, the gram stain was done. As a result, *Lactobacillus* could be still alive at 50°C. Therefore, *Lactobacillus* heated at 70°C and 100°C was used for the optimization of prodigiosin production in *Streptomyces coelicolor*.

Production of PG on liquid YEME medium

The experiment was carried out on YEME broth medium because the PG production did not occur on GYP broth medium in both the pure culture of *S. coelicolor* and the culture inoculated with the dead cells of *L. rhamnosus*. The samples started to be collected after the cultures incubated for 24 hours. Remarkably, The prodigiosin production occurred after second day of incubation (Fig. 1). After sixth day, the prodigiosin production reduced (Fig. 2).

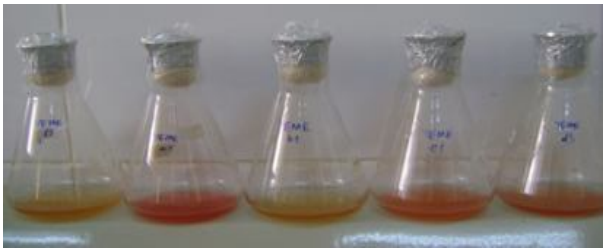

Fig. 1: The second day of *S. coelicolor* incubation. (1): *S. coelicolor* without *L. rhamnosus*; (2), (3): *S. coelicolor* was inoculated with 50µL, 100µL of *L. rhamnosus* cells heated at 70°C; (3), (4): *S. coelicolor* was inoculated with 50µL, 100µL of *L. rhamnosus* cells heated at 100°C.

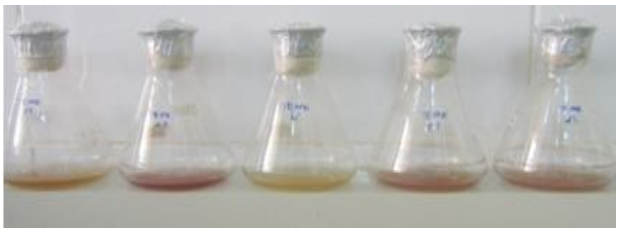

Fig. 2: The sixth day of *S. coelicolor* incubation. (1): *S. coelicolor* without *L. rhamnosus*; (2), (3): *S. coelicolor* was inoculated with 50µL, 100µL of *L. rhamnosus* cells heated at 70°C; (4), (5): *S. coelicolor* was inoculated with 50µL, 100µL of *L. rhamnosus* cells heated at 100°C.

The PG yields in 50µL of heat-killed cells at 70°C of *L. rhamnosus* were extracted and measured. The results were presented in the table 1. As seeing in figure 1, figure 2 and table 1, the prodigiosin production showed the strongest when using 50 µL of *L. rhamnosus* cells heated at 70°C. The tested treatments of heated *L. rhamnosus* gave the significant difference at the second day for incubation volume of 20 ml when the analyses were performed by one-way ANOVA. The 50µl of heated *L. rhamnosus* at 70°C gave the best result and reached the maximum of 9.79 mg/L. The red color in the flask reduced when using 100 µL of *L. rhamnosus* cells heated at 70°C. There was a binding of *Lactobacillus* component with the factors relating to PG synthesis. The increasing the amount of *Lactobacillus rhamnosus* caused the lower PG. However, the PG production reduced when *Streptomyces* incubated with the heated *L. rhamnosus* cells at 100°C. However, in terms of interpretation at molecular level, the mechanism of this kind of stimulation is not fully understood. Some suggestions were given that the dead cells have lysed compounds that may play role as precursors for the product. That component should be done so far.

Table. 1: Production of prodigiosin (mg/L) by *S. coelicolor* without and with heated *L. rhamnosus* cells at 70°C.

Time (day)	*S. coelicolor*	*S. coelicolor* with heated cells at 70°C of *L. rhamnosus*
1	0.4 ± 0.07	1.16 ± 0.23
2	1.43 ± 0.22	9.79 ± 1.68
3	0.51 ± 0.11	5.22 ± 0.62
4	0.27 ± 0.04	2.72 ± 0.31
5	0.34 ± 0.14	2.64 ± 0.37
6	0.31 ± 0.12	2.13 ± 1.24

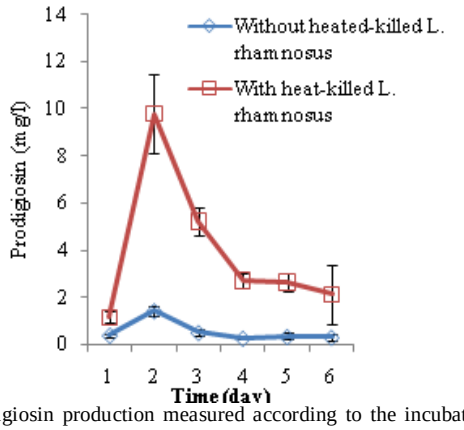

Fig. 3: Prodigiosin production measured according to the incubation time of *Streptomyces coelicolor*. The blue line showed the prodigiosin in *Streptomyces coelicolor*. The red line showed the prodigiosin produced in *Streptomyces coelicolor* after incubated with heated *L. rhamnosus* at 70°C.

Usually, when *L. rhamnosus* was heated, the outer membrane as lipopolysaccharide were still stable and could affect on PG. The *L. rhamnosus* was heated at 100°C which reduced the PG in the comparison with the *L. rhamnosus* was heated at 70°C. It was meant that there were some components being stable only at 70°C affecting on PG production. After the sixth day of incubation, the PG production decreased in the incubation with 100µl of heated *L. rhamnosus* cells at 70°C as well as with 50µL, 100µL of heated *L. rhamnosus* cells at 100°C. It was meant that the *L. rhamnosus* compounds affected at the early stage of PG production. Therefore, the heated *L. rhamnosus* compounds might interact to other compounds produced in *Streptomyces* in the sixth day condition. *Lactobacillus rhamnosus* is gram-positive organisms are characterized by thick peptidoglycan layer which mainly contained N-acetyl glucosamine compounds. This compound may play role in triggering antibiotic production in *Streptomyces* (Tsao *et al.*, 1985). Besides, many compounds from biological sources including protein, cell wall fragments, yeast extract or carbohydrates were used to enhance the production of secondary metabolites in microbial, whole-plant, and plant cell cultures (Radman *et al.*, 2003).

As presented in figure 3 and figure 4, the culture started producing PG on the second day of incubation. It reached its maximum of 1.43mg/L on the second day and the maximum wet biomass concentration of 27.65g/l was obtained on the third day. The culture inoculated with 50 µl of heated *L. rhamnosus* cells at 70°C rose to the maximum of 9.79 mg/L on the second day, higher than *Streptomyces* production at six – fold.

In the figure 4, the biomass was maximum at 25.5g/L on the third day of incubation. However, the prodigiosin production highly obtained in the second day. Obviously, the heated *L. rhamnosus* affected on the early stage of *S. coelicolor*. After that, prodigiosin was down because the unstability of the heated *L. rhamnosus* compounds or there were the interaction of *S.coelicolor* secondary metabolism which reduced the prodigiosin production. Many mechanisms should be propagated so far.

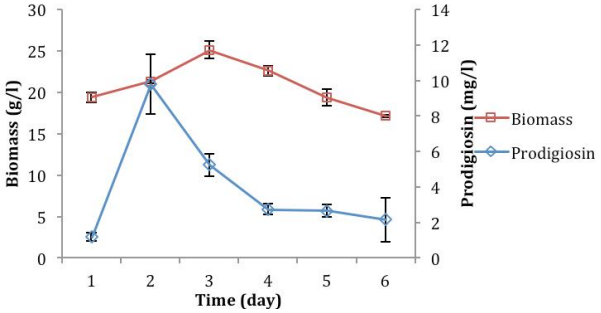

Fig. 4: The biomass and prodigiosin production of *Streptomyces coelicolor* when incubated with heated *L. rhamnosus* at 70°C.

CONCLUSION

The heated cells of *L. rhamnosus* at 70°C stimulated the production of prodigiosin on *S. coelicolor* at the early stage of *S. coelicolor*. Using the dead cells make more convenient than others compounds that need to be extracted from cells or cell walls due to its simple, less costly. In addition, this study may lead a strategy in order to enhance the production of other antibiotics. However, which components that cause the enhancement of PG production are not known yet. Many studies need to be conducted so far to achieve the knowledge about those components and the mechanism of this interaction. In the industrial point of view, the study played a role as a preliminary step and should be scaled up so far.

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